Nicotine suppresses acute colitis and colonic tumorigenesis associated with chronic colitis in mice

Shusaku Hayashi, Takayuki Hamada, Syed Faisal Zaidi, Momoe Oshiro, Jaemin Lee, Takeshi Yamamoto, Yoko Ishii, Masakiyo Sasahara, and Makoto Kadowaki

1Division of Gastrointestinal Pathophysiology, Institute of Natural Medicine, University of Toyama, Toyama, Japan; and 2Department of Pathology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

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Am J Physiol Gastrointest Liver Physiol 307: G968–G978, 2014. First published September 25, 2014; doi:10.1152/ajpgi.00346.2013.—Ulcerative colitis is a chronic inflammatory disease that frequently progresses to colon cancer. The tumor-promoting effect of inflammation is now widely recognized and understood. Recent studies have revealed that treatment with nicotine ameliorates colitis in humans and experimental murine models, whereas the effect of nicotine on colitis-associated colorectal tumorigenesis remains unclear. In the present study, we examined the effect of nicotine on the development of acute colitis and colitis-associated cancer (CAC). The acute colitis model was induced by treatment with 3% dextran sulfate sodium (DSS) for 7 days, whereas the CAC model was induced by a combination of azoxymethane and repeated DSS treatment. Nicotine and a selective agonist of the α7-nicotinic acetylcholine receptor (α7-nAChR) reduced the severity of DSS-induced acute colonic inflammation. In addition, the suppressive effect of nicotine on acute colitis was attenuated by an antagonist of α7-nAChR. Furthermore, nicotine inhibited the IL-6 production of CD4 T cells in the DSS-induced inflamed colonic mucosa. We found that nicotine significantly reduced the number and size of colonic tumors in mice with CAC. Nicotine markedly inhibited the elevation of TNF-α and IL-6 mRNA as well as phospholipid signal transducer and activator of transcription (Stat) 3 expression in the colons of the tumor model mice. These results demonstrate that nicotine suppresses acute colitis and colitis-associated tumorigenesis, and this effect may be associated with the activation of α7-nAChR. Furthermore, it is presumed that nicotine downregulates the expression of inflammatory mediators such as IL-6/Stat3 and TNF-α, thereby reducing the colonic tumorigenesis associated with chronic colitis.

α7-nicotinic acetylcholine receptor; dextran sulfate sodium-induced colitis; CD4 T cells

INFLAMMATORY BOWEL DISEASES (IBDs), such as Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders that affect the gastrointestinal tract (33). UC is characterized by pathological mucosal damage and ulceration, which extend proximally from the rectum to varying degrees (33). The incidence of UC in Western countries and Japan has risen dramatically in recent decades. UC typically presents as a relapsing disorder marked by attacks of bloody mucoid diarrhea that can persist for months, only to recur after an asymptomatic interval of months to years (15). Repetitive relapses and remissions frequently cause epithelial dysplasia and can eventually progress to cancer (50). Indeed, the risk of colorectal cancer development in UC patients is much higher than that in the general population (30). Long-standing UC predisposes patients to the development of colitis-associated cancer (CAC), which is the major cause of death in UC patients (13). Thus, effective therapies based on an understanding of the pathogenesis of CAC at the molecular and cellular levels are required to prevent CAC development in UC patients.

Recent studies indicate that inflammation plays an important role in promoting cancer development (21). Proinflammatory cytokines are expressed in the tumor microenvironment, and such cytokine signalings are also important for cancer development through the activation of downstream transcription factors (10). Among these cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6 can activate nuclear factor (NF)-κB and signal transducer and activator of transcription (Stat) 3, respectively, and both the TNF-α/NF-κB and IL-6/Stat3 pathways have been shown to be critical for the development of CAC (21, 34). Immune cells, such as lymphocytes and macrophages, in the gut microenvironment are recognized as a major source of TNF-α and IL-6 (19, 40, 48). Furthermore, micro-RNA (miR)-21, a downstream factor of the IL-6/Stat3 pathway, has been reported as an oncogenic miRNA in various carcinomas (32, 46).

Recently, the cholinergic anti-inflammatory pathway has been regarded as a novel therapeutic target (2) due to the effectiveness of targeted treatments in animal models of sepsis (7), postoperative ileus (11), and colitis (17, 18). Indeed, there is an epidemiological relationship between IBD and smoking (4). UC patients with a history of smoking usually acquire their disease after they have stopped smoking (41). UC patients who smoke intermittently often experience an improvement in their colitis symptoms during the period when they smoke (44). In contrast, CD patients experience severe disease when smoking, requiring an immediate and complete smoking cessation (23). The administration of nicotine in transdermal patches ameliorates inflammation associated with UC patients (35). Several studies have suggested that the anti-inflammatory action of acetylcholine is mediated by the homomeric α7-subunit of the nicotinic acetylcholine receptor (α7-nAChR), which is known to be located on immune cells, such as mast cells (26), macrophages (51), dendritic cells, and CD4 T cells (28). Furthermore, the administration of nicotine and specific agonists of α7-nAChR shows anti-inflammatory effects in various experimental animal models (1, 17, 27). However, the ameliorative effect of nicotine and the activation of α7-nAChR in the colitis model remain controversial (47). Furthermore, it is unclear whether nicotine has a beneficial effect on the development of chronic inflammation-associated colorectal cancer.
In the present study, we examined the effect of nAChR activation on dextran sulfate sodium (DSS)-induced acute colitis and CAC in mice, and we investigated the underlying mechanisms.

**METHODS**

**Animals.** Male BALB/c mice (8–10 wk old) were purchased from Japan SLC (Shizuoka, Japan). All mice were housed in the experimental animal facility at the University of Toyama and were provided free access to food and water. The Animal Experiment Committee at the University of Toyama approved all of the animal care procedures and experiments (authorization no. A2012INM-2).

**Induction of acute colitis by DSS.** For acute colitis induction, mice were administered 3% DSS (36–50 kDa; MP Biomedicals, Santa Ana, CA) in their drinking water for 7 days (38). To assess the severity of colitis, body weight, stool consistency, and blood in the stool were monitored daily. The disease activity index (DAI) was the sum of two parameters as follows: diarrhea (0, normal; 1, loose stools; 2, watery diarrhea) and blood in the stool (0, normal; 1, slight bleeding; 2, gross bleeding). The experimenter measuring DAI was blinded to the treatment given to the mice.

**Histological study.** The distal part of colon was fixed in 4% paraformaldehyde embedded in paraffin and sliced into 5-μm-thick sections. The sections were then routinely stained with hematoxylin and eosin (H&E). H&E-stained sections were scored for inflammation and crypt damage as described previously (8). To exclude bias, histological scores were determined in a masked manner. Two independent parameters were measured as follows: extent of inflammation (0, none; 1, mild; 2, moderate; 3, severe) and extent of crypt damage (0, none; 1, damage to the basal one-third portion; 2, damage to the basal two-thirds portion; 3, damage to the entire crypt with surface epithelium intact; 4, erosion). Histological score was assessed as the sum of two parameters.

**CAC model.** Mice were administered azoxymethane (AOM) (12 mg/kg; Sigma-Aldrich, St. Louis, MO) intraperitoneally. Five days later, mice were administered 2% DSS in their drinking water for 5 days, followed by 16 days of regular water. This cycle was repeated a total of three times. Body weight was measured every other day, and the colonic mucosa was monitored using a mouse endoscopy system (AE-C1; AVS, Tokyo, Japan). On day 90 after the AOM administration, the mouse colon was excised for macroscopic evaluation, histological analysis, and biological analysis. The visible tumors (>1 mm in the major axis) were counted in the mid to distal colon of each mouse. After macroscopic assessment, proximal and distal parts of colon were fixed in 4% paraformaldehyde embedded in paraffin and sliced into 5-μm-thick sections. The sections were then routinely stained with H&E. Histological assessment was performed by two pathologists (Ishii and Sasahara) blinded to the treatment given to the mice. Dysplastic or adenocarcinoma lesions were determined by previously established criteria (9).

**Determination of myeloperoxidase activity.** Myeloperoxidase (MPO) activity was measured in the mouse colon as described previously. Briefly, the animals were killed 7 days after DSS treatment, and the colons were excised. After the tissue was rinsed with cold saline, the whole colon was weighed and homogenized on ice in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0). The homogenized samples were subjected to three cycles of freeze-thawing and then centrifuged at 2,000 rpm for 30 min at 4°C. The MPO activity in the supernatant was determined by adding 100 μL of the supernatant to 2.9 ml of 0.5 M o-dianisidine hydrochloride in 10 mM phosphate buffer (pH 6.0) containing 0.0005% (wt/vol) hydrogen peroxide. The changes in the absorbance of each sample were recorded at 460 nm using a spectrophotometer (UV160A; Shimadzu, Kyoto, Japan). MPO activity was expressed as units per wet weight of colonic tissue in grams.

**Determination of TNF-α and IL-6 mRNA expression.** Total RNA was extracted from the colon using Sepasol RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions. Reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan) and random primers followed by real-time PCR. Real-time PCR amplification of IL-6, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using SYBR Premix EX Taq (Takara Bio). The following primer pairs were used: TNF-α, forward 5’-AAAGCTGTAGC-CCACGTGTA-3’ and reverse 5’-GGCAACACTAGT GTGTT-GCTTGGT-3’; IL-6, forward 5’-CACCTTCAAGTGCCGAG-GCTTA-3’ and reverse 5’-GCAAATGTACCATGGTGTTCTTAC-3’; and GAPDH, forward 5’-TGA CCCAGTCCATGGCATT-3’ and reverse 5’-GACGGACACATTTGGGTTAG-3’. Real-time PCR was performed using the Takara TP800 (Takara Bio). The PCR reaction conditions consisted of 10 s at 95°C followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. Target mRNA levels were normalized to those of GAPDH as an internal control in each sample. The results are expressed as ratios relative to the average of the control group.

**Isolation of lamina propria CD4 T cells.** Lamina propria mononuclear cells (LPMCs) were isolated from the mouse colons as described below. The colons were removed, opened longitudinally, and washed of fecal contents with ice-cold RPMI-1640 (Wako, Osaka, Japan). The colons were then cut into small pieces that were stirred at 37°C for 20 min in RPMI-1640 containing 2% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 0.5 mM EDTA and washed two times with RPMI-1640. This process was repeated without EDTA. The pieces were incubated in 37°C for 20 min in RPMI-1640 containing 200 U/ml collagenase (Wako), and digested tissues were collected and washed with RPMI-1640. This process was repeated three times, and cells were pooled. Cell suspensions were collected, passed through a strainer (70 μm), and washed with RPMI-1640. Isolated cells were suspended in 40% Percoll (Sigma-Aldrich), layered onto 75% Percoll, and centrifuged at 2,000 rpm for 20 min. LPMCs were recovered from the interphase and washed two times with RPMI-1640. LP CD4 T cells were purified by negative selection from LPMCs using the BD IMag Mouse CD4 T Lymphocyte Enrichment Set (BD Biosciences, San Jose, CA). For determination of αS-nAChR mRNA expression, total RNA was extracted from isolated CD4 T cells using a RNAeasy Plus Micro kit (Qiagen, Valencia, CA). RT-PCR was performed as described above. The primer pairs of αS-nAChR were used as follows: forward 5’-‘AACCATGGCCGTAGGACA-3’ and reverse 5’-CTCAGCCCAAGCAGCATGAA-3’. A portion of the PCR mixture was electrophoresed on a 2% agarose gel containing 0.1% ethidium bromide in Tris-borate-EDTA buffer and photographed.

**Measurement of IL-6 protein level.** Isolated CD4 T cells (2 × 10^5) were seeded on 24-well culture plates (BD Biosciences) in RPMI-1640 supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich) and stimulated with Dynabeads Mouse T-Activator CD3/CD28 (Gibco) for 48 h at 37°C in a humidified incubator with 5% CO₂. Culture supernatants were collected and stored at −80°C until IL-6 measurement. IL-6 in culture supernatants were detected by a cytometric beads array kit (BD Biosciences) according to the manufacturer’s instructions. Samples were analyzed with a FACSCanTo II flow cytometer (BD Biosciences).

**Determination of pStat3 expression by Western blotting.** The distal colon was removed, washed with ice-cold PBS, and homogenized in lysis buffer (pH 7.4) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Nacalai Tesque). After centrifugation at 20,000 g for 30 min at 4°C, the protein concentrations in the supernatants were determined using a BCA protein assay kit (Pierce, Rockford, IL). An appropriate volume of the sample was mixed with an equal volume of sample buffer [pH 6.8; 125 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 1% mercaptoethanol, and 0.02% bromphenol blue] and heated at 95°C for 5 min. The samples (20 μg/lane) were then subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred electrophoretically to PVDF membranes (Millipore, Billerica, MA). The membranes were incubated with rabbit anti-pStat3
antibody or rabbit anti-Stat3 antibody (Cell Signaling Technology, Danvers, MA) and treated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology). Immune complexes were visualized using the enhanced chemiluminescence detection system (GE Healthcare Japan, Tokyo, Japan) and photographed (ImageQuant LAS4000; GE Healthcare Japan). The expression level of pStat3 protein was determined densitometrically with ImageQuant TL (GE Healthcare Japan).

**Isolation of colonic epithelial cells.** Colonic epithelial cells were isolated from the mouse colons according to the previous report (19). The colons were removed, opened longitudinally, and washed of fecal contents with ice-cold PBS. The colons were then cut into small pieces that were stirred in Hank’s balanced salt solution containing 30 mM EDTA at 37°C for 10 min. The supernatant was collected and centrifuged, and then the resulting pellet was washed with ice-cold PBS. Isolated colonic epithelial cells were incubated with 100 ng/ml IL-6 (PeproTech, Rocky Hill, NJ) at 37°C for 30 min. The expression of pStat3 protein in the colonic epithelial cells was determined by Western blotting.

**Determination of micro-RNA-21 expression.** Total RNA was extracted from the colon using Sepasol RNA I Super (Nacalai Tesque) according to the manufacturer’s instructions. cDNA was synthesized from 500 ng of total RNA using the miScript II RT Kit (Qiagen) followed by real-time PCR. Real-time PCR amplification of mir-21 and U6 was performed using the miScript SYBR Green PCR Kit (Qiagen). The PCR cycling conditions were as follows: 95°C for 15 min followed by 40 cycles at 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. For detection of mature miR-21, the miScript Primer Assay for mmu-mir-21 (Qiagen) was used according to the manufacturer’s protocol. The miScript primer sets for U6 (Qiagen) were used for normalization.

**Statistical analyses.** The data are presented as means ± SE. Statistical analyses were performed using Student’s unpaired t-test and one-way repeated-measures ANOVA followed by Dunnett’s multiple-comparison test. Values of \( P < 0.05 \) were considered to be significant.

**RESULTS**

**Effect of nicotine on the development of DSS-induced acute colitis.** We examined the effect of nicotine on the development of DSS-induced acute colitis in mice. Animals treated with 3% DSS (36–50 kDa) for 7 days developed symptoms of colitis. Diarrhea was first observed on day 4 after the start of treatment, followed by rectal bleeding and body weight loss (Fig. 1, A and B). Nicotine (3 mg/kg), which was administered subcutaneously one time daily for 7 days during DSS treatment, reduced the severity of DSS-induced colitis on day 7 (Fig. 1B). Nicotine treatment significantly attenuated the severity of DSS-induced colitis (Fig. 1F).

**Effect of nicotine on the development of DSS-induced acute colitis.** Nicotine, which was administered subcutaneously one time daily for 7 days during DSS treatment, reduced the severity of TNF-α and IL-6 mRNA expression in the colon of the mice. The elevated MPO activity was significantly suppressed by nicotine (DSS-nicotine mice; 1,076.7 ± 445.4).

**Effect of nicotine on the development of CAC in mice.** We examined the expression of α7-nAChR mRNA in CD4 T cells isolated from colonic LP of mice. As shown in Fig. 3A, a clear expression of α7-nAChR mRNA was observed in CD4 T cells of normal mice and DSS-induced colitis mice.

To identify the effect of nicotine on IL-6 production in CD4 T cells of inflamed colonic mucosa, we analyzed IL-6 protein level in the culture supernatant of colonic CD4 T cells isolated from normal or colitis mice. The purified CD4 T cells of inflamed colonic mucosa spontaneously produced a large amount of IL-6 (1,039.3 ± 51.3 pg/ml) compared with CD4 T cells of normal colonic mucosa (223.8 ± 7.9 pg/ml) (Fig. 3B). Nicotine (1 and 3 mM) significantly inhibited the IL-6 production of CD4 T cells in a concentration-dependent manner, with the IL-6 level being 78 and 53% of the vehicle treatment, respectively. Furthermore, IL-6 production of CD4 T cells in the inflamed mucosa was enhanced by stimulation with anti-CD3/CD28 antibody. Nicotine (3 mM) also significantly suppressed augmented IL-6 production to 72% of vehicle treatment.

**Effect of nicotine on the development of CAC in mice.** We used the well-established AOM/DSS mouse model of CAC. Endoscopic and macroscopic analyses revealed that AOM/DSS treatment (Fig. 4A) induced the development of multiple tumors in the mid to distal colon of mice (CAC-vehicle mice, 17.3 ± 2.0, Fig. 4, B–D). Nicotine treatment significantly suppressed the multiplicity of the macroscopically visible tu-

Fig. 1. Effect of nicotine on the development of dextran sulfate sodium (DSS)-induced acute colitis in mice. Colitis was induced in mice by daily treatment at a 3% DSS solution in drinking water for 7 days. Body weight (A), disease activity index (B), and colon length (C) are shown (n = 8–12 mice). D: representative images of hematoxylin and eosin staining are shown. Scale bar, 100 μm. E: histological scoring of DSS-induced colitis (n = 4–6). F: effect of nicotine on TNF-α (left) and IL-6 (right) mRNA expression induced by DSS treatment in the mouse colon. The data are presented as means ± SE of 4–6 mice and are representative of one of two (F) or three (G) independent experiments. *Significant difference from normal at \( P < 0.05 \). †Significant difference from vehicle at \( P < 0.05 \).
EFFECT OF NICOTINE ON COLITIS-ASSOCIATED CANCER

A

Body weight (% of initial weight)

0 1 2 3 4 5 6 7
Day

Normal
3% DSS
Vehicle
Nicotine 3 mg/kg

B

Disease activity index

0 1 2 3 4 5 6 7
Day

Normal
3% DSS
Vehicle
Nicotine 3 mg/kg

C

Colon length (cm)

0 2 4 6 8 10 12
Normal Vehicle Nicotine

D

Histological score

0 1 2 3 4 5 6 7
Normal Vehicle Nicotine

E

Relative TNF-α mRNA level

0 1 2 3 4 5 6 7 8 9 10
Normal Vehicle Nicotine

F

MPO activity (units/g tissue)

0 500 1000 1500 2000 2500 3000
Normal Vehicle Nicotine

G

Relative IL-6 mRNA level

0 100 200 300 400 500 600
Normal Vehicle Nicotine
mors induced by AOM/DSS treatment (CAC-nicotine mice, 8.8 ± 1.1, Fig. 4D), and these tumors were reduced in size compared with those in CAC- and vehicle-treated mice. Histopathological analysis of the distal colon revealed no difference in the incidence of adenocarcinomas between CAC- and vehicle-treated mice and CAC- and nicotine-treated mice, whereas tumors from CAC- and nicotine-treated mice were markedly smaller in size compared with those from CAC- and vehicle-treated mice (Fig. 5). Furthermore, we observed pre-cancerous lesions, such as dysplasia in the proximal colon of all CAC- and vehicle-treated mice (n = 6, Fig. 5), although there were no macroscopically visible tumors (Fig. 4C). In contrast, only one mouse out of eight CAC- and nicotine-treated mice exhibited an incidence of dysplasia in the proximal colon (n = 8, Fig. 5).

**Fig. 2.** Involvement of α7-nicotinic acetylcholine receptor (α7-nAChR) in the suppressive effect of nicotine on the development of DSS-induced acute colitis in mice. Colitis was induced in mice by daily treatment with a 3% DSS solution in drinking water for 7 days. Nicotine or an α7-nAChR agonist (GTS-21; 10 mg/kg) was administered sc one time daily for 7 days, whereas an α7-nAChR antagonist (methyllycaconitine; 10 mg/kg) was administered sc 30 min before the administration of nicotine. Body weight (A) and disease activity index (B) are shown (n = 8). The data are presented as means ± SE. *Significant difference from normal at P < 0.05. †Significant difference from vehicle at P < 0.05. #Significant difference from nicotine at P < 0.05.

**Fig. 3.** Effect of nicotine on the interleukin (IL)-6 production in CD4 T cells of inflamed colonic mucosa. CD4 T cells were isolated from the colons (8 pooled colons) of normal or DSS-induced colitis mice using the IMag system. A: the expression of α7-nAChR mRNA in colonic CD4 T cells of normal or DSS-induced colitis mice. Water (W) was amplified as negative control template. M, molecular mass markers. B: the concentration of IL-6 in the culture supernatants of CD4 T cells. CD4 T cells were stimulated with Dynabeads Mouse T-Activator CD3/CD28 for 48 h in the absence or presence of nicotine (1 or 3 mM), and the culture supernatants were analyzed for IL-6 protein by a cytometric beads array kit. The data are presented as means ± SE of three independent experiments. *Significant difference from normal at P < 0.05. †Significant difference from vehicle (nonstimulation) at P < 0.05. #Significant difference from vehicle (anti-CD3/CD28) at P < 0.05.
the normal mice (Fig. 6, B and C), and its expression was significantly suppressed by treatment with nicotine (CAC-nicotine, Fig. 6, B and C). Furthermore, we examined the effect of IL-6 on phosphorylation of Stat3 in the colonic epithelial cells. We observed that the expression of phosphorylated Stat3 was increased by IL-6 stimulation (Fig. 6D).

Effect of nicotine on the expression of miR-21 in the colon of mice with CAC. In addition, we examined the expression level of miR-21 in the colon of mice with CAC. The miR-21 expression level in the colon of CAC- and vehicle-treated mice was significantly increased (2-fold) compared with that in normal mice (Fig. 7). This enhancement of miR-21 expression was markedly suppressed by the treatment with nicotine (Fig. 7).

**DISCUSSION**

A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors (10, 21). Indeed, recent studies using an experimental CAC mouse model have revealed that inflammatory pathways, such as the TNF-α/NF-κB and IL-6/Stat3 cascades, play a pivotal role in the development of colonic inflammation-associated tumorigenesis (21), suggesting that inhibitors of proinflammatory cytokines might have therapeutic potential for CAC (49). Recently, the cholinergic anti-inflammatory pathway has been found to control the inflammatory diseases, including IBD (6). In this pathway, acetylcholine, the principal neurotransmitter of the vagus nerve, stimulates α7-nAChR to inhibit cytokine release from immune cells (51). Nicotine, a nonspecific agonist of nAChR, can pharmacologically mimic these anti-inflammatory effects (11). We therefore hypothesized that the activation of α7-nAChR by nicotine can inhibit colitis and colitis-associated tumorigenesis through the exertion of anti-inflammatory effects on pathogenic immune cells in the colon. In the present study, we demonstrated that nicotine suppressed the development of chronic inflammation-associated colorectal cancer as well as acute colitis in mice.

Using the DSS-induced colitis model, we showed the ameliorative effect of nicotine against the development of acute
colonic inflammation; furthermore, we found that the activation of α7-nAChR is involved in this process. Nicotine treatment significantly attenuated the symptoms of DSS-induced colitis accompanied by diarrhea, rectal bleeding, and colon shortening. We observed that immune cell infiltration in the colonic mucosa induced by DSS treatment was decreased by nicotine administration. Immune cells, which infiltrate the inflamed gut of IBD patients, can produce various cytokines that trigger inflammatory responses (3). Among them, TNF-α and IL-6 are positively correlated with the disease activities of IBD, and their production returns to normal in the absence of severe inflammation in IFN-γ-deficient mice (24) and IL-17A-deficient mice (25), indicating the importance of T cell responses and IL-6 production in CD4 T cells of the inflamed colonic mucosa. These findings strongly suggest that the activation of α7-nAChRs on lymphocytes is involved in the regulation of proinflammatory cytokine production (16). In the present study, we demonstrated that nicotine directly inhibited IL-6 production in activated CD4 T cells, resulting in the suppression of colitis severity.

A noteworthy finding in this study was that nicotine significantly suppressed the number and size of tumors in CAC mice. The AOM/DSS model is a well-established and widely used mouse model for CAC development, and it mimics UC-related colon cancer (39). Using this murine model, we evaluated the incidence and multiplicity of colonic tumors on day 90 after AOM administration because we were able to observe visible tumors at day 80 by endoscopic investigation. Nicotine had a potent suppressive effect on tumor formation in the mid to distal colon induced by AOM/DSS treatment, although adenocarcinomatous lesions developed in vehicle- and nicotine-treated mice in the CAC model. Interestingly, we observed precancerous lesions in the proximal colon of all vehicle-treated mice in the CAC model, whereas the incidence of precancerous lesions was markedly decreased in nicotine-treated mice. These results suggest that nicotine suppresses both tumor promotion and tumor initiation because of its anti-inflammatory effects.
Several proinflammatory molecules have been implicated in the development of CAC (21, 39). The first critical genetic evidence that inflammatory cells serve as a source of tumor-promoting cytokines was obtained in a mouse model of CAC; NF-κB inactivation in the myeloid cells of these mice reduced tumor growth and blocked the production of IL-6 and other cytokines in response to colitis (19). IL-6 is one of the major NF-κB-inducible cytokines, and it is important for immune responses, cell survival, apoptosis, and proliferation (29). IL-6 protein and mRNA are often increased in the serum and tumor tissues of humans and mice with various cancers, including colon cancer (22). Indeed, AOM/DSS-induced CAC development is significantly suppressed in IL-6-deficient mice (20). In the present study, we demonstrated the increased expression of IL-6 mRNA and the drastic suppression of this increase by nicotine treatment in the colon of CAC mice. Stolfi et al. (48) reported that tumor-infiltrating macrophages and T cells are the major source of IL-6 during the tumorigenesis in the lamina propria of CAC mice. Additionally, the proportion of colonic CD4 T cells is associated with the tumor incidence and severity of inflammation in CAC mice because CD4 T cells are involved in the growth of CAC (48). We found that α7-nAChR mRNA was expressed in CD4 T cells of the lamina propria isolated from both normal and colitis mice. Thus, these findings suggest that nicotine suppresses the production of IL-6 by CD4 T cells through the activation of α7-nAChR and eventually prevents the development of CAC. Furthermore, we found that DSS treatment caused a dramatic elevation of IL-6 mRNA expression compared with TNF-α in the colon of acute colitis mice and that nicotine almost completely inhibited the upregulation of IL-6. We therefore focused on the IL-6-dependent pathway as a target for the suppressive effect of nicotine on...
CAC development. In the CAC model, IL-6 exerts a tumor-promoting effect primarily via Stat3 because cell type-specific inactivation of Stat3 in intestinal epithelial cells results in reduced carcinogenesis in mice treated with AOM and DSS (5, 20). Stat3 plays a pivotal role in the regulation of transcriptional programs for carcinogenesis, cell survival, proliferation, angiogenesis, and tumor-induced immunosuppression (54). In the present study, we demonstrated that nicotine suppressed the upregulation of phosphorylated Stat3 in the colon of mice with CAC. The number and size of AOM/DSS-induced colonic tumors are significantly increased in gp130757F/F mice, in which IL-6-dependent Stat3 signaling is constitutively activated (5). These results indicate that Stat3 activation in epithelial cells plays a critical pathological role in the promotion of intestinal tumorigenesis. Indeed, we observed that the expression of phosphorylated Stat3 was upregulated by IL-6 stimulation. Taken together, these results indicate that nicotine treatment inhibits Stat3 activation through the reduction of IL-6 production in colonic CD4 T cells, thereby resulting in tumor suppression.

Recently, Putoczki et al. (42) have reported that IL-11, an IL-6 family cytokine, is a potent driver of Stat3 activation during gastrointestinal tumor progression in a CAC mouse model similar to that used in the present study. However, the remaining tumors in the colon and remaining Stat3 activation in the intestinal epithelial cells have been observed in IL-6-deficient mice and IL-11 receptor-deficient mice, and CAC and Stat3 activation is almost completely absent in the colon of double-deficient mice (IL-6 and IL-11 receptor) (42). Although the precise individual and synergistic roles of IL-6 family cytokines on CAC formation are not fully understood, these findings suggest that IL-6 activates Stat3 in the epithelial cells together with other IL-6 family cytokines during the development of CAC. In the future, more studies are needed to unravel the involvement of other cytokines, including IL-11, in the suppressive effect of nicotine/α7-nAChR against the development of CAC.

Furthermore, we found that the miR-21 level in the colon of mice with CAC was increased, and nicotine completely attenuated this response. The expression of various miRNAs is considered to be altered in IBD, and, notably, active inflammation in UC is closely associated with increased expression of miR-21 (52). miR-21 has been described as an oncogenic miRNA that exhibits anti-apoptotic activity in various carcinomas (46). In addition, miR-21 expression can be induced by IL-6 in a Stat3-dependent manner, and miR-21 promotes the survival of myeloma cells (32). Thus, elevated miR-21 may have a causative role in CAC. In a human cohort study, Schetter et al. (45) reported that the expression of IL-6 mRNA was significantly increased in colon adenocarcinomas from 196 patients, whereas the expression of TNF-α mRNA was significantly decreased. Additionally, the expression of miR-21 was associated with IL-6 expression but not TNF-α expression. Taken together, our findings suggest that nicotine inhibits the upregulation of miR-21 via the suppression of the IL-6/Stat3 pathway and attenuates tumor promotion in CAC mice.

TNF-α, one of key regulators of inflammatory responses, can further augment NF-κB activation in various cell types and induce the expression of inflammatory factors, including cyclooxygenase-2, IL-6, and TNF-α itself (31). Using the CAC model, Popivanova et al. (40) have demonstrated that tumors fail to develop in TNF-α receptor-deficient mice because of the reduction of inflammatory cell infiltration. Similarly, we showed that TNF-α mRNA expression was enhanced in the colon of CAC mice, and we demonstrated the suppressive effect of nicotine on this enhanced expression. These findings indicate that the nicotine-induced inhibition of TNF-α production in inflammatory cells also contributes to the suppressive effect of nicotine on CAC development.

In the present study, we demonstrated that the exogenous nicotine suppressed the development of colitis and CAC in mice. Until now, anti-inflammatory effects of nicotinic agonists have been demonstrated repeatedly in several inflammation models. However, the role of endogenous acetylcholine in the inflammation was poorly understood. Recently, we have reported that the activation of α7-nAChRs alleviated the allergic symptoms in the food allergy mice through the inhibition of mucosal mast cell functions (26, 53). In addition, mucosal mast cells were located in close proximity to nerve fibers of intrinsic cholinergic enteric neurons in the colon of food allergy mice (53). These findings suggest that the cholinergic enteric neuroimmune interaction via α7-nAChRs on immune cells plays an important role in the controlling pathological immune activation to restore the homeostasis in the intestine. However, it remains unclear whether cholinergic nerve fibers interact with colonic CD4 T cells in the inflamed colonic mucosa. Furthermore, in 2011, Rosas-Ballina et al. (43) reported that acetylcholine synthesized by CD4 T cells is greatly involved in suppressing TNF-α production in macrophages during vagus nerve stimulation, suggesting that acetylcholine secreted from CD4 T cells acts on immune cells through an autocrine and/or paracrine pathway to modulate immune function. Thus, further detailed studies are needed to clarify the mechanism of the underlying cholinergic anti-inflammatory pathway in the intestinal inflammation.
Given the findings of the present study, we conclude that nicotine suppresses DSS-induced acute colitis and colonic tumorigenesis associated with chronic colitis in mice. By activating α7-nAChR on CD4 T cells in the colonic mucosa, nicotine inhibits the production of IL-6, resulting in the suppression of Stat3 activation and miR-21 expression. However, clinical application of nicotine is difficult because nicotine has adverse effects. Thus, we would like to clarify more detailed mechanisms underlying the anti-inflammatory effect of α7-nAChR activation and demonstrate the possibility of α7-nAChR-specific agonist as a therapeutic target in CAC.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.H. and M.K. conception and design of research; S.H., T.H., S.F.Z., M.O., Y.L., and M.S. performed experiments; S.H., T.H., S.F.Z., and T.Y. analyzed data; S.H., J.L., Y.I., M.S., and M.K. interpreted results of experiments; S.H. and S.F.Z. prepared figures; S.H. and M.K. drafted manuscript; S.H. and M.K. edited and revised manuscript; S.H. approved final version of manuscript.

REFERENCES


